Benzodiazepines and Metabolites from Blood and Urine by LC/MS (MRM)

1 Introduction

Benzodiazepines are one of the most commonly prescribed classes of drugs in the United States. They are also frequently abused. Therapeutic blood concentrations vary for benzodiazepines, depending on whether they are considered high dose benzodiazepines (e.g., diazepam) or low dose benzodiazepines (e.g., triazolam). Benzodiazepines are usually excreted into the urine as glucuronide metabolites, and may persist in the urine for days after administration due to elimination half-lives that may exceed 24 hours.

2 Scope

This procedure allows for the screening and confirmation of the following benzodiazepines in blood and urine: 7-aminoclonazepam, 7-aminoflunitrazepam, α-hydroxyalprazolam, α-hydroxymidazolam, α-hydroxytriazolam, alprazolam, chlordiazepoxide, clonazepam, desalkylflurazepam, diazepam, flunitrazepam, flurazepam, lorazepam, midazolam, n-desmethylflunitrazepam, nordiazepam, oxazepam, temazepam, and triazolam. It also provides for quantitation of these compounds in blood (except flurazepam). This document applies to Chemistry Unit case working personnel who perform toxicology analyses.

3 Principle

Biological specimens are qualitatively assayed and/or quantified for benzodiazepines and their metabolites. Specimens are mixed with deuterated internal standards. Proteins are precipitated from blood before extraction. Both blood and urine samples are extracted using solid phase extraction (SPE). Analysis of extracts is by liquid chromatography/tandem mass spectrometry in the multiple reaction monitoring mode (LC/MS(MRM)).

4 Specimens

This procedure uses 0.2 mL of blood (in duplicate for quantitation[1]) or 0.4 mL of urine.

5 Equipment/Materials/Reagents

a. Routine laboratory supplies, including calibrated pipettes, disposable culture tubes, test

[1] If case history, screening results, or other previous analysis indicates that the concentration of a benzodiazepine may be above the linear range of the method, the sample may be prediluted before extraction.

tube racks, graduated cylinders, etc.

b. Methanol (Optima Grade)

c. Deionized water (18 MΩ)

d. 1:1 Methanol:Water

e. Zinc sulfate heptahydrate

f. Zinc Sulfate (0.2 M): Combine 50 mL deionized water and 5.75 g of zinc sulfate heptahydrate in a 100 mL volumetric flask. Mix until dissolved. Bring to the mark with deionized water. Store in glass at room temperature. Stable for at least 6 months.

g. Zinc Sulfate in Methanol: Combine 80 mL methanol and 20 mL zinc sulfate (0.2 M) in a volumetric flask and mix well. Mix well. Store in glass at room temperature. Stable for at least 2 months.

h. Potassium dihydrogen phosphate

i. Disodium hydrogen phosphate (anhydrous)

j. Potassium Phosphate Buffer: Add 9.07 g potassium dihydrogen phosphate to a 1 L volumetric flask and bring to the mark with deionized water. Store refrigerated in glass or plastic. Stable for at least three months.

k. Sodium Phosphate Buffer: Add 11.6 g disodium hydrogen phosphate (anhydrous) to a 1 L volumetric flask and bring to the mark with deionized water. Store refrigerated in glass or plastic. Stable for at least three months.

l. Sorensen Buffer (pH 7.4): Add sodium phosphate buffer to the potassium phosphate buffer until the pH reads 7.4 with a pH meter. Store refrigerated in glass or plastic. Stable for at least three months.

m. β-Glucuronidase - (>100,000 u/mL β-glucuronidase activity; from Red Abalone, *H. Rufescena*; available from Kura Biotec)

n. Ammonium acetate (99.999% purity)

o. Acetic acid, glacial (17 M, ACS grade)

p. Ammonium Acetate Buffer (0.5 M; pH 5):
Add 3.854 g ammonium acetate to a 100-mL volumetric flask containing about 75 mL deionized water. Mix well to dissolve. Add glacial acetic acid until pH registers between

4.5 and 5.5. Bring to volume with deionized water and mix well. Store refrigerated in glass or plastic. Stable at least three months.

q. Vortexer

r. Centrifuge

s. Evaporator with nitrogen

t. SPE manifold

u. pH meter

v. Oasis HLB 6 cc (500 mg) LP SPE cartridges

w. Ammonium hydroxide, concentrated (15 M) (ACS grade)

x. Methanol:Water:Ammonia (40:60:0.5):
Combine 40 mL methanol, 60 mL deionized water and 0.5 mL ammonium hydroxide and mix well. Store at room temperature in glass. Prepare fresh daily.

y. Methylene chloride (Optima grade)

z. Isopropanol (HPLC grade)

aa. Methylene Chloride:Isopropanol (75:25):
Combine 75 mL methylene chloride and 25 mL isopropanol and mix well. Store at room temperature in glass. Stable for at least two months.

bb. Water:Acetonitrile (90:10):
Combine 90 mL deionized water and 10 mL acetonitrile (Optima grade) and mix well. Store at room temperature in glass. Stable for at least three months.

cc. Centrifuge tube filters (0.45 micron, Nylon)

dd. Ammonium formate

ee. Formic acid

ff. Mobile Phase A (5 mM Ammonium Formate with formic acid; pH~3.5): Add 0.3153 g ammonium formate to a 1 L volumetric flask. Add approximately 800 mL deionized water and mix well. Add 1 mL formic acid, and QS with deionized water. Store in glass at room temperature. Stable for at least one week.

gg. Mobile Phase B (Acetonitrile with 0.1% Formic Acid): Combine 1 mL formic acid and 1000 mL acetonitrile and mix well. Store in glass at room temperature. Stable for at least two months.

hh. ABI 6500+ (or equivalent) Liquid Chromatograph/Mass Spectrometer equipped with Analyst software and a Phenomenex Kinetex XB-C18 (or equivalent) analytical column (150 mm x 2.1mm x 2.6 µ)

6 Standards and Controls

a. Standard and Control Stock Solutions (1.0 mg/mL) of the following may be purchased from Cerilliant (Round Rock, TX), Lipomed or an equivalent supplier. The materials used to prepare the standard stock solutions will be from a different source than the materials used to prepare the control stock solutions. Solutions may be in methanol or acetonitrile, and will be stored according to the manufacturer's recommendations. Stability is determined by the manufacturer.

7-aminoclonazepam	clonazepam	n-desmethylflunitrazepam
7-aminoflunitrazepam	desalkylflurazepam	nordiazepam
α-hydroxyalprazolam	diazepam	oxazepam
α-hydroxymidazolam	flunitrazepam	temazepam
α-hydroxytriazolam	flurazepam	triazolam
alprazolam	lorazepam	
chlordiazepoxide	midazolam	

b. Internal Standard Stock Solutions (0.1 mg/mL) of the following may be purchased from Cerilliant (Round Rock, TX) or an equivalent supplier. Solutions may be in methanol or acetonitrile, and will be stored according to the manufacturer's recommendations. Stability is determined by the manufacturer.

7-aminoclonazepam-d_4	chlordiazepoxide-d_5	midazolam-d_4
7-aminoflunitrazepam-d_7	clonazepam-d_4	n-desmethylflunitrazepam-d_4
*α-hydroxyalprazolam-d_5	desalkylflurazepam-d_4	nordiazepam-d_5
α-hydroxymidazolam-d_4	diazepam-d_5	oxazepam-d_5
α-hydroxytriazolam-d_4	flunitrazepam-d_7	temazepam-d_5
alprazolam-d_5	lorazepam-d_4	triazolam-d_4
oxazepam glucuronide-d_5	*used for flurazepam	

c. Internal Standard Intermediate Solution (5 µg/mL):
Add 0.25 mL of each Internal Standard Stock Solution to a 5-mL volumetric flask and bring to the mark with methanol. Store in the freezer. Stable for at least 2 years.

d. Internal Standard Working Solution (500 ng/mL):
Combine 0.1 mL of the Internal Standard Intermediate Solution (5 µg/mL) and 0.9 mL methanol. Prepare fresh daily.

e. Calibration Scheme

This procedure uses a multi-point calibration curve for the analyte(s) of interest following the *Guidelines for Toxicological Quantitations* standard operating procedure (Tox 101).

Preparation of High Calibration Solution			
Starting Solution	1	mg/mL	stock solution(s)
Starting Solution Aliquot	0.025	mL	
Diluent Volume	25	mL	methanol, volumetric flask
Resulting Concentration	1	µg/mL	storage: freezer; stability: ≥ 1 year

Preparation of Low Calibration Solution			
Starting Solution	1	µg/mL	High Calibrator Solution
Starting Solution Aliquot	0.5	mL	
Diluent Volume	10	mL	methanol, volumetric flask
Resulting Concentration	50	ng/mL	storage: freezer; stability: ≥ 1 year

Calibrator Level	Low Cal Spike (µL)	High Cal Spike (µL)	Resulting Concentration, ng/mL (in 0.2mL of blood)
1	10		2.5
2	20		5
3	100		25
4	200		50
5		20	100
6		30	150
7		50	250
8		75	375

f. Control Scheme

Negative Control Blood is purchased from Cliniqa or another approved vendor. Storage and

stability determined by manufacturer. A Negative Control Blood sample will be extracted and analyzed with every blood assay.

At least one Positive Control Blood Sample will be analyzed with each blood assay. For quantitative analyses, both levels of Positive Control Blood Samples will be analyzed in duplicate.

Preparation of High Control Solution			
Starting Solution	1	mg/mL	stock solution(s)
Starting Solution Aliquot	0.025	mL	
Diluent Volume	25	mL	methanol, volumetric flask
Resulting Concentration	1	µg/mL	storage: freezer; stability: ≥ 1 year

Preparation of Low Control Solution			
Starting Solution	1	µg/mL	High Control Solution
Starting Solution Aliquot	0.5	mL	
Diluent Volume	10	mL	methanol, volumetric flask
Resulting Concentration	50	ng/mL	storage: freezer; stability: ≥ 1 year

Control Level	Low Control Spike (µL)	High Control Spike (µL)	Resulting Concentration, ng/mL (in 0.2mL of blood)
Negative	0	0	0
Low	20	0	5
High	0	50	250

g. Hydrolysis Check Internal Standard Intermediate Solution (2.0 µg/mL d_5-Oxazepam equivalent): To a 10-mL volumetric flask, add 0.322 mL of the d_5-Oxazepam Glucuronide Stock Standard. Bring to volume with acetonitrile. Store frozen in glass. Stable for at least 6 months.

h. Hydrolysis Check Internal Standard Working Solution (200 ng/mL d5-Oxazepam equivalent): Combine 0.1 mL of the Hydrolysis Check Internal Standard Intermediate Solution (2.0ug/mL) and 0.9 mL deionized water. Prepare fresh daily.

i. Negative Control Urine:
Prepared in-house or purchased from an appropriate vendor. Stable for 6 months when

refrigerated. A Negative Control Urine sample will be extracted and analyzed with every urine assay.

j. Positive Control Urine Samples:

Prepare the Working Standard Control Solution (50 ng/mL) by adding 0.5 mL High Control Solution (1 µg/mL) to a 10-mL volumetric flask and diluting with 1:1 Methanol:Water.

1. Low Positive Control Urine (1 ng/mL):
   Mix 0.020 mL of the Benzodiazepine Working Standard Control Solution (50 ng/mL) with 1.0 mL of Negative Control Urine. Mix well before withdrawing 0.4 mL for analysis. Prepare fresh.
2. High Positive Control Urine (10 ng/mL):
   Mix 0.010 mL of the High Control Solution (1 µg/mL) with 1.0 mL of Negative Control Urine. Mix well before withdrawing 0.4 mL for analysis. Prepare fresh.
3. Hydrolysis Control Urine (10 ng/mL):
   Add 0.020 mL of the Hydrolysis Check Internal Standard Working Solution to a 0.4 mL aliquot of the high positive control urine. (This results in a 10 ng/mL concentration of d_5-oxazepam.) Prepare fresh.

At least one Positive Control Urine Sample will be analyzed with every urine assay.

k. LC/MS Performance Standard (5 ng/mL): Add 5 µL of the Benzodiazepine Intermediate Standard Calibrator Solution (1 µg/mL) and 10 µL of the Internal Standard Working Solution to 1 mL of Water:Acetonitrile (90:10). Store in refrigerated autosampler tray for up to one week or prepare fresh daily.

l. This procedure uses a multi-point calibration curve for the analyte(s) of interest following the *Quality Control for Toxicology Examinations* (TOX101).

7 Sampling

Representative portions of the specimens are obtained. See TOX101 for further details.

8 Procedure

Appendix 1 contains an abbreviated version of this procedure. This form may be used at the bench by the authorized individual performing the procedure.

8.1 Sample Preparation for Blood Specimens

a. Prepare blood calibrators and controls as directed in Section 6 above. A second analyte

source is used for the controls (see TOX101 for further guidance).

b. Pipet 0.2 mL of each case blood sample into a properly labeled 12 x 75 mm test tube. (Blood samples will be prepared in duplicate for quantitation and may have to be diluted for benzodiazepine concentrations that are above the linear range of the procedure.)

c. Add 0.020 mL of the Internal Standard Working Solution (500 ng/mL) to each sample and vortex well.

d. Add 2 mL zinc sulfate in methanol to each blood sample. Allow to sit for 1 minute, then vortex.

e. Centrifuge samples for 5 minutes at 3000 rpm.

f. Transfer supernatant to a new, properly labeled 16 x 100 mm test tube.

g. Concentrate samples to ~0.4 mL under nitrogen at 60°C.

h. Add 5.5 mL of Sorenson buffer to each tube.

i. Vortex and centrifuge samples for 1 minute at 3000 rpm.

8.2 Sample Preparation for Urine Specimens

a. Prepare urine controls as directed in Section 6 above.

b. Pipet 0.4 mL of each case urine sample into a properly labeled 16 x 100 mm test tube.

c. Add 0.010 mL of the Internal Standard Working Solution (500 ng/mL) to each sample (except the hydrolysis control sample) and vortex well.

d. Add 0.6 mL Ammonium Acetate Buffer (0.5 M, pH 5) and 0.1 mL β-glucuronidase.

e. Vortex, cap, and incubate 30 minutes at 68°C.

f. Cool to room temperature.

g. Add 2 mL zinc sulfate in methanol to each urine sample. Allow to sit for 1 minute, then vortex.

h. Centrifuge samples for 5 minutes at 3000 rpm.

i. Transfer supernatant to a new, properly labeled 16 x 100 mm test tube.

j. Concentrate samples to ~1.0 mL under nitrogen at 60°C.

k. Add 5 mL of Sorenson buffer to each tube.

l. Vortex and centrifuge samples for 1 minute at 3000 rpm.

8.3 Solid Phase Extraction (applicable to blood and urine samples)

a. Pre-rinse SPE extraction cartridge (Oasis HLB) by adding 2 mL of methanol.

b. Condition cartridge with 3 mL of deionized water.

c. Load sample on SPE cartridge.

d. Wash cartridge with 2 mL of Methanol:Water:Ammonia (40:60:0.5).

e. Dry cartridge at full vacuum for 15 minutes. (Use vacuum manifold; positive pressure source not shown to dry effectively.

f. Elute with 5 mL Dichloromethane:Isopropanol (75:25) under gravity.

g. Evaporate eluent to dryness at 60°C under nitrogen.

h. Reconstitute blood extracts with 0.25 mL Water:Acetonitrile (90:10). Vortex. Reconstitute urine extracts with 0.1 mL of Water:Acetonitrile (90:10). Vortex.

i. Filter samples through 0.45 micron filters.

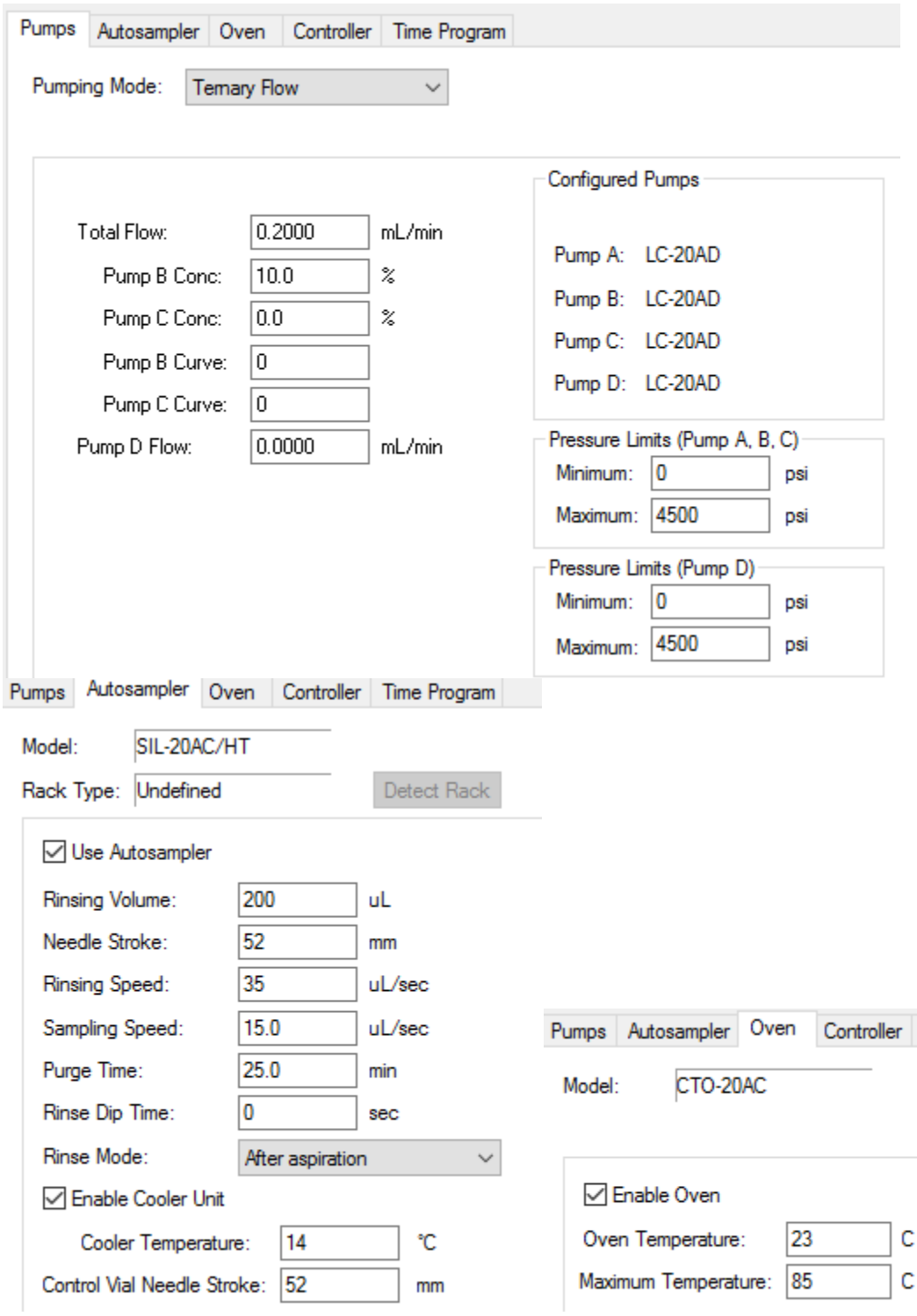
j. Analyze 5 µL of the LC/MS Performance Standard to verify that the instrument is operating properly and that retention times have not shifted outside of the analytes' multiple reaction monitoring (MRM) windows.

k. Analyze extracts following the instrumental conditions in Section 9 below.

9 Instrumental Conditions

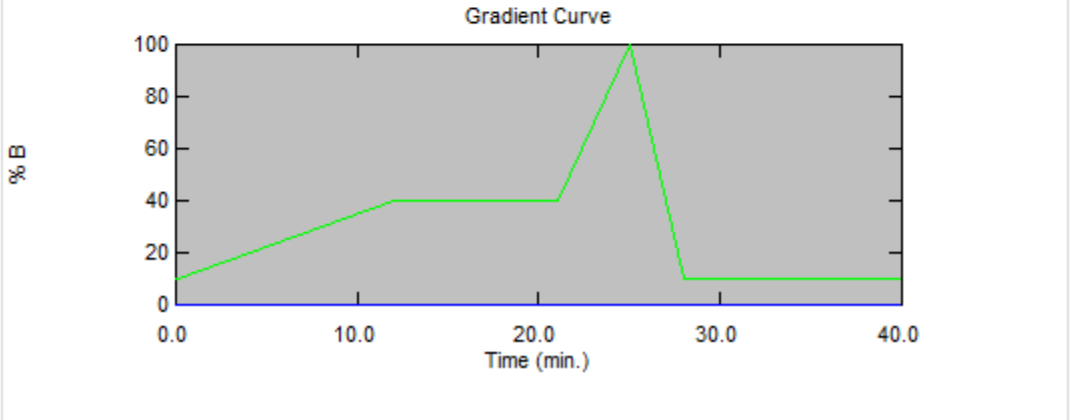
Appendix 1 contains an abbreviated version of the instrumental conditions in this procedure. This form may be used at the bench by the authorized individual performing the procedure; recording reagents/material used in the unit chemical inventory system is also acceptable.

9.1 LC Parameters

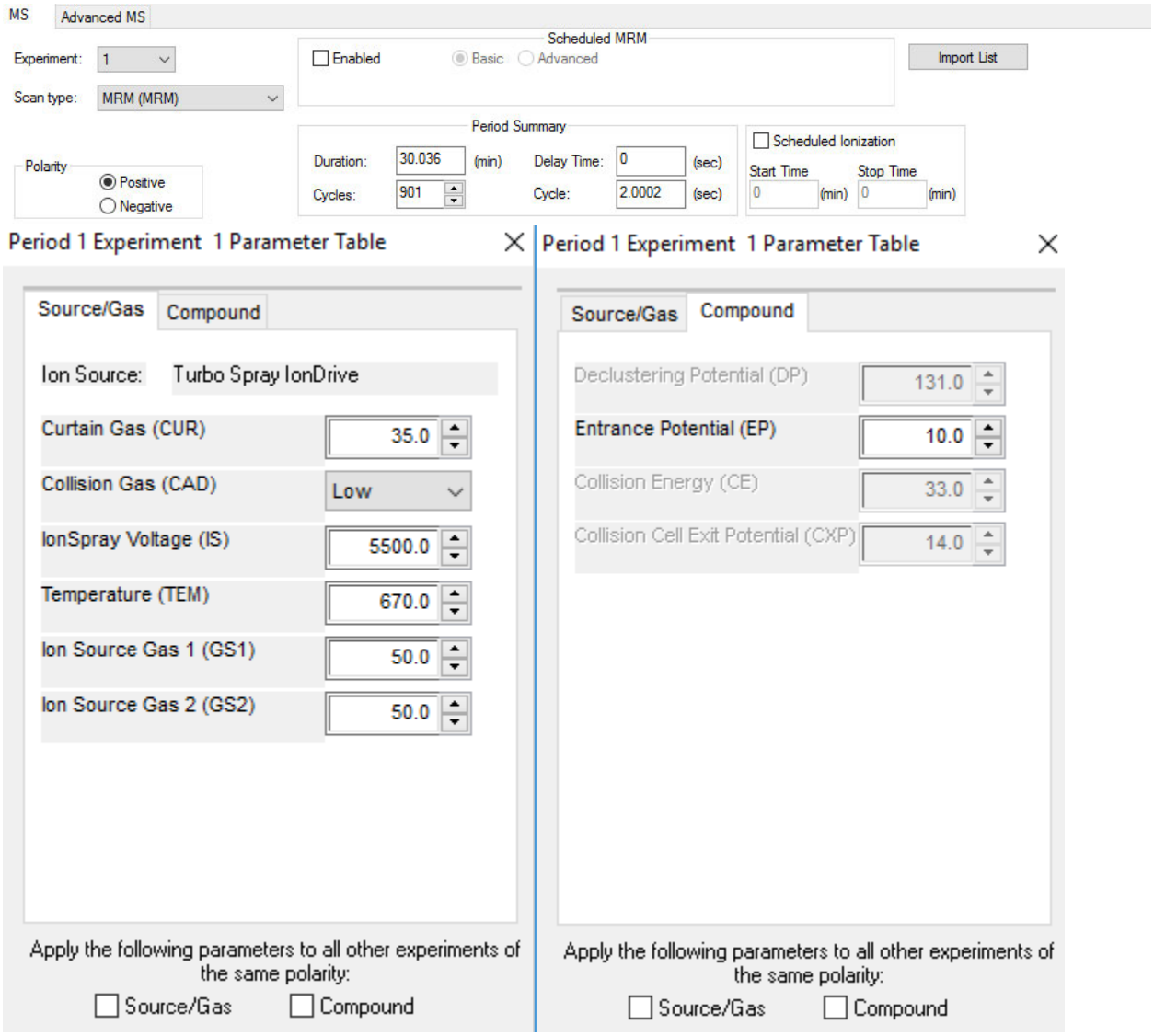


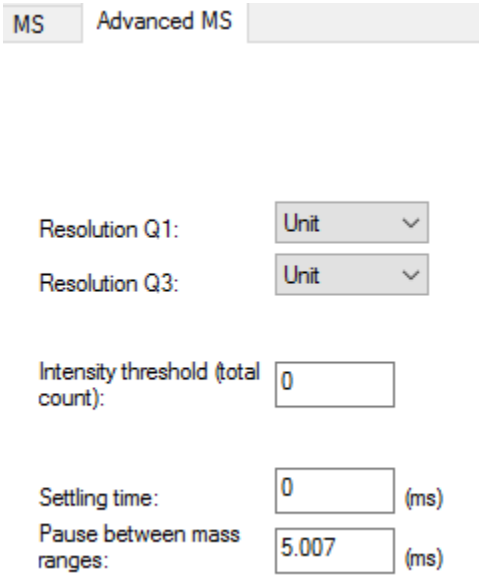
Pumps | Autosampler | Oven | Controller | Time Program

	Time	Module	Event	Parameter
1	0.10	Pumps	Total Flow	0.2
2	0.10	Pumps	Pump B Conc.	10
3	12.00	Pumps	Pump B Conc.	40
4	21.00	Pumps	Pump B Conc.	40
5	25.00	Pumps	Pump B Conc.	100
6	28.00	Pumps	Pump B Conc.	10
7	40.00	Pumps	Pump B Conc.	10
8	40.01	Controller	Stop	
9				



9.2 Mass Spectral Parameters





9.3 Analyte Specific Parameters

Q1 Mass (Da)	Q3 Mass (Da)	Dwell Time (msec)	Analyte	DP (volts)	CE (volts)	CXP (volts)
286.233	222.2	12.4	7_aminoclonazepam_1	131	33	14
286.233	250.2	12.4	7_aminoclonazepam_2	131	29	14
286.233	195.2	12.4	7_aminoclonazepam_3	131	43	12
288.219	222.2	12.4	7_aminoclonazepam_4	100	33	12
290.218	226.2	12.3	7_aminoclonazepam_d4	126	35	14
284.285	227.3	14.1	7_aminoflunitrazepam_1	151	35	14
284.285	240.3	14.1	7_aminoflunitrazepam_2	151	45	14
284.285	135	14.1	7_aminoflunitrazepam_3	151	35	14
291.285	138	13.9	7_aminoflunitrazepam_d7	121	39	16
325.25	297.2	19.4	alpha_hydroxyalprazolam_1	106	35	16
325.25	216.1	19.4	alpha_hydroxyalprazolam_2	106	53	24
327.247	299.2	19.4	alpha_hydroxyalprazolam_3	181	35	16
330.273	302.3	19.2	alpha_hydroxyalprazolam_d5	136	37	16
342.211	203	16.6	alpha_hydroxymidazolam_1	71	35	12
342.211	168.1	16.6	alpha_hydroxymidazolam_2	71	49	18
344.216	205	16.6	alpha_hydroxymidazolam_3	111	35	12
346.232	203	16.6	alpha_hydroxymidazolam_d4	151	37	12
359.211	176	19.3	alpha_hydroxytriazolam_1	151	37	20
359.211	331.3	19.3	alpha_hydroxytriazolam_2	151	37	18
359.211	239.2	19.3	alpha_hydroxytriazolam_3	151	61	14

361.195	333.2	19.3	alpha_hydroxytriazolam_4	100	37	18
363.229	176	19.2	alpha_hydroxytriazolam_d4	176	37	12
309.266	205.1	23.2	Alprazolam_1	130	45	12
309.266	281.2	23.2	Alprazolam_2	130	35	16
311.237	283.2	23.2	Alprazolam_3	126	35	16
314.253	279.3	23	Alprazolam_d5	131	35	16
300.217	227.1	14.9	chlordiazepoxide_1	91	33	18
300.217	241.051	14.9	chlordiazepoxide_2	91	31	16
300.217	165.1	14.9	chlordiazepoxide_3	91	63	18
305.133	232.1	14.8	chlordiazepoxide_d4	81	33	14
316.194	270.2	22.5	clonazepam_1	146	35	16
316.194	214.1	22.5	clonazepam_2	146	49	12
318.184	272.2	22.5	clonazepam_3	141	35	14
320.238	274.2	22.3	clonazepam_d4	191	35	16
289.173	226.2	24.3	Desalkylflurazepam_1	36	39	12
289.173	179.1	24.3	Desalkylflurazepam_2	36	59	20
289.173	140.004	24.3	Desalkylflurazepam_3	36	39	16
291.132	226.2	24.3	Desalkylflurazepam_4	111	39	14
293.205	230.2	24.1	Desalkylflurazepam_d4	56	39	14
285.086	193.2	26.9	diazepam_1	126	47	22
285.086	154.1	26.9	diazepam_2	126	35	18
287.214	193.1	26.9	diazepam_3	141	43	22
290.203	198.2	26.8	diazepam_d5	46	45	24
314.22	268.2	25.3	flunitrazepam_1	106	35	14
314.22	239.2	25.3	flunitrazepam_2	106	45	14
314.22	183.1	25.3	flunitrazepam_3	106	67	20
321.295	275.3	24.9	flunitrazepam_d7	101	37	16
388.577	315.2	17.1	flurazepam_1	56	31	18
388.577	288.2	17.1	flurazepam_2	56	35	16
390.313	317.2	17.1	flurazepam_3	121	31	18
321.203	275.1	21.5	Lorazepam_1	91	33	14
321.203	229.2	21.5	Lorazepam_2	91	43	12
323.186	277.2	21.5	Lorazepam_3	96	29	16
327.203	281.1	21.4	Lorazepam_Cl_d4	146	27	6
326.205	249.1	16.8	Midazolam_1	176	51	14
326.205	291.3	16.8	Midazolam_2	176	37	16
326.205	222.3	16.8	Midazolam_3	176	63	28
328.19	291.2	16.8	Midazolam_4	150	37	16
330.237	253.1	16.7	midazolam_d4	126	53	14

300.173	198.1	20.4	N_desmethylflunitrazepam_1	91	53	22
300.173	254.2	20.4	N_desmethylflunitrazepam_2	91	37	14
300.173	225.1	20.4	N_desmethylflunitrazepam_3	91	47	12
304.211	258.2	20.3	N_desmethylflunitrazepam_d4	116	33	16
271.258	208.3	23.5	Nordiazepam_1	141	39	12
271.258	165.3	23.5	Nordiazepam_2	141	37	20
271.258	140.025	23.5	Nordiazepam_3	141	37	16
276.382	213.2	23.1	Nordiazepam_d5	26	39	12
287.231	241.3	20.2	Oxazepam_1	161	31	14
287.231	231.2	20.2	Oxazepam_2	161	31	12
289.213	243.2	20.2	Oxazepam_3	106	29	14
292.196	246.2	20.1	Oxazepam_d5	101	33	14
301.266	255.1	25.3	Temazepam_1	101	29	22
301.266	177	25.3	Temazepam_2	101	51	20
303.251	257.2	25.3	Temazepam_3	71	29	16
306.215	260.2	25	Temazepam_d5	81	33	16
343.213	239.2	24.3	Triazolam_1	36	55	12
343.213	308.3	24.3	Triazolam_2	36	35	18
345.212	241.1	24.3	Triazolam_3	36	55	14
347.216	243.3	24.1	Triazolam_d4	166	57	14

10 Decision Criteria

10.1 LC/MS Performance Standard Decision Criteria

Peaks should show good chromatographic fidelity, with reasonable peak shape, width, and resolution. The authorized individual should ensure that the peaks entirely elute within their MRM windows, and adjust the MRM window times, if necessary.

10.2 Unknown Sample Decision Criteria

The following criteria are used as guidelines in determining the acceptability of the data produced in this assay.

10.2.1 Batch Acceptance

No analytes of interest should be detected in the Negative Control. For this purpose, analytes of interest are defined as any analytes that are being reported for this batch.

Each of the analytes in the Positive Control should be detected in the LC/MS data. High and Low

Positive Controls should fall within ±20% of the target value. See the *Guidelines for Toxicological Quantitations* standard operating procedure (Tox 101) for further guidance.

There should be a peak for d5-oxazepam in the Hydrolysis Check Positive Control Urine. This peak area should approximate the area of the oxazepam peak (within ±50%).

10.2.2 Unknown Sample Criteria

Each of the Internal Standards should be detectable in the LC/MS data.

10.2.2.1 Chromatography

The peak of interest should show good chromatographic fidelity, with reasonable peak shape, width, and resolution. In order to be determined acceptable, a chromatographic peak in an unknown sample should compare favorably to a chromatographic peak of the same analyte in a known sample analyzed on the same system in the same or subsequent analytical runs. Additionally, the following two criteria should be met.

10.2.2.2 Retention Time

The retention time of the peak should be within ± 2% of the retention time (relative or absolute, as appropriate) obtained from injection of a reference standard, an extracted Positive Control, or an appropriate deuterated analog.

10.2.2.3 Signal-to-Noise

To justify the existence of a peak, its baseline signal to peak-to-peak noise ratio should exceed 10 when using the Analyst software. Further, the baseline signal for the peak of interest should be at least 10 fold greater than that for any observed peak at similar retention time in a Negative Control or solvent blank injected just prior to the sample.

10.2.2.4 Mass Spectrometry

At least three independent MS/MS experiments are conducted for each analyte. Two ion ratios are calculated for each analyte. The mass spectrum of the analyte of interest should match that of a reference standard, extracted calibrator, or an extracted Positive Control. See the *Guidelines for Comparison of Mass Spectra* standard operating procedure (TOX104) for further guidance.

11 Calculations

$1/x^2$ weighting is used for all calibration curves. See *Quality Control for Toxicology Examinations* (TOX101) for acceptable practices in calculating quantitative results.

12 Measurement Uncertainty

The critical sources of measurement uncertainty in this procedure include:

- historical random uncertainty of repeated measurements
- accuracy of the pipette used to deliver the sample
- accuracy of the pipette used to deliver the calibrators
- uncertainty in the concentration of the calibration standards
- precision of the delivery of internal standard

When quantitative results are included in an FBI Laboratory Report, the measurement uncertainty will be estimated and reported following the *Chemistry Unit Procedures for Estimating Measurement Uncertainty* (CUQA 13). Information used to derive uncertainty measurements will be tracked in an electronic database.

13 Limitations

(Values from original validation; see validation binder for relevant updates)

a. Limit of Detection:
   1. Blood: 1.25 ng/mL (or lower)
   2. Urine: 0.5 ng/mL (or lower)

b. Limit of Quantitation: 2.5 ng/mL

c. Accuracy (as % bias; n=15 for all values in table):

	Bias (%; at 5 ng/mL)	Bias (%; at 100 ng/mL)	Bias (%; at 250 ng/mL)
7-aminoclonazepam	-3.63	2.06	3.65
7-aminoflunitrazepam	2.71	5.89	1.32
α-OH midazolam	3.93	3.76	0.44
α-OH alprazolam	3.83	3.88	-1.22
diazepam	5.21	4.54	-2.80
clonazepam	0.39	3.69	1.41
alprazolam	6.84	6.45	-1.85
chlordiazepoxide	0.40	2.11	-2.69
flunitrazepam	1.23	2.09	-0.17
desalkylflurazepam	2.71	2.42	0.36
lorazepam	2.04	2.89	0.69
flurazepam	2.89	-1.79	-0.79
n-desmethylflunitrazepam	0.09	2.11	-0.78
midazolam	0.03	2.17	1.01
nordiazepam	2.29	4.72	-2.76
oxazepam	0.52	0.97	-1.00

temazepam	5.29	5.26	-2.21
triazolam	7.57	5.43	-3.56
α-OH triazolam	2.31	4.94	-0.14

d. Precision (as both repeatability and intermediate precision; n=15 for all values in tables):

	Repeatability (%; at 5 ng/mL)	Repeatability (%; at 100 ng/mL)	Repeatability (%; at 250 ng/mL)
7-aminoclonazepam	7.20	9.71	8.96
7-aminoflunitrazepam	7.29	6.49	11.09
α-OH midazolam	5.45	3.22	4.26
α-OH alprazolam	4.95	2.78	4.22
diazepam	5.26	2.34	3.33
clonazepam	4.72	2.47	3.86
alprazolam	4.83	3.66	2.85
chlordiazepoxide	3.70	3.14	4.38
flunitrazepam	4.54	3.02	2.76
desalkylflurazepam	4.79	4.03	4.01
lorazepam	6.79	3.28	3.12
flurazepam	9.55	5.69	3.70
n-desmethylflunitrazepam	3.94	3.62	3.76
midazolam	4.31	3.17	3.75
nordiazepam	5.00	2.79	3.73
oxazepam	5.08	2.65	3.08
temazepam	4.32	2.70	2.96
triazolam	4.59	3.17	1.92
α-OH triazolam	4.67	3.37	4.00

	Intermediate Precision (%; at 5 ng/mL)	Intermediate Precision (%; at 100 ng/mL)	Intermediate Precision (%; at 250 ng/mL)
7-aminoclonazepam	10.29	9.71	9.17
7-aminoflunitrazepam	7.29	6.49	11.65
α-OH midazolam	6.45	5.25	5.74
α-OH alprazolam	5.72	5.04	5.37
diazepam	5.36	3.94	5.95
clonazepam	4.91	4.05	5.37
alprazolam	5.63	5.04	6.66
chlordiazepoxide	4.45	5.04	6.63
flunitrazepam	5.04	4.74	4.47
desalkylflurazepam	5.44	4.64	6.60
lorazepam	7.67	7.38	7.53
flurazepam	10.45	7.50	6.29
n-desmethylflunitrazepam	5.40	4.50	4.81
midazolam	5.29	5.09	6.33

nordiazepam	5.40	5.20	5.40
oxazepam	5.68	3.29	3.16
temazepam	5.52	5.84	5.45
triazolam	5.11	5.21	5.34
α-OH triazolam	4.91	5.83	6.22

e. Cautionary Statement: Oxazepam may be unstable in methanol.

14 Safety

Take standard precautions for the handling of chemicals and biological materials. Refer to the *FBI Laboratory Safety Manual* for guidance.

15 References

Baselt, R.C. *Disposition of Toxic Drugs and Chemicals in Man*, 9th ed.; Biomedical Publications, Seal Beach, California, 2011.

Quality Control for Toxicology Examinations (TOX101); FBI Laboratory Chemistry Unit - Toxicology SOP Manual.

Chemistry Unit Procedures for Estimating Measurement Uncertainty (CUQA 13); FBI Laboratory Chemistry Unit Quality Assurance and Operations Manual.

Guidelines for Comparison of Mass Spectra (TOX104); FBI Laboratory Chemistry Unit – Toxicology SOP Manual.

FBI Laboratory Safety Manual.

Rev. #	Issue Date	History
6	04/01/19	Removed "subunit" (header, 15). Updated CUQA title in Reference Section and 12. Updated blood reconstitution volume to 0.25mL (8.3-h, Appendix). Made format/typo corrections on page 1 of Appendix. Updated enzyme to >100,000 (5-m) to account for product description.
7	06/01/21	Adjusted Scope (Section 2) to specify flurazepam analysis is qualitative only. Corrected SRM to MRM in Section 3 and title as well as on bench notes. Updated Section 5-hh. to include new instrument. Corrected Section 6 typos in item numbering and clarified urine control preparation. Section 6-b: clarified internal standard for flurazepam. In Section 6-l, removed unnecessary phrase. Updated phrase in Section 7. Changed text to "authorized individual" in Section 8, 9 and 10.1. In Section 8.1-a, updated control preparation language. Reformatted Section 9 and removed some parameters from instrument bench notes; added phrase to Section 9 indicating that recording information in the chemical inventory database is also acceptable. Updated TOX101 title in Sections 6-l, 11 and 15. In Section 10.2.2.4, removed Table 2 and updated text. In Section 13 added clarifying limitation statement.

Approval

Redact - Signatures on File

Toxicology
Technical Leader: Date: 06/01/2021

Chemistry Unit Chief: Date: 06/01/2021

Appendix 1: Abbreviated version of the Benzodiazepine Procedure for bench use (page 1-3)

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Appendix 1: Abbreviated version of the Benzodiazepine Procedure for bench use (page 1-3) (continued)

Redact - Form on File

Appendix 1: Abbreviated version of the Benzodiazepine Procedure for bench use (page 1-3) (continued)

Redact - Form on File